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## Unsaturated Fatty Acids of Butterfat

This study was undertaken to characterize more completely the unsaturated fatty acids of butterfat. The presence of  $C_{10}$  to  $C_{18}$  monoethenoid acids was confirmed; the  $C_{12}$  and  $C_{14}$  were predominantly the *cis*-form, while the  $C_{16}$  and  $C_{18}$  acids had both *cis* and *trans* double bonds. The nonconjugated dienoic acids were found to be a mixture of *cis-cis* and either *cis-trans* or *trans-trans* isomers. Conjugated dienoic acids were identified as *cis-trans* and *trans-trans* isomers. The trienoic, tetraenoic, and pentaenoic acids had the all-*cis* configuration.

THE COMPOSITION OF BUTTERFAT has been the subject of literally hundreds of investigations in the past 50 years. The presence of the normal straight chain saturated fatty acids from  $C_4$  to  $C_{20}$ , with an even number of carbon atoms, has been fairly well established. However, in recent years, Shorland and coworkers have reported the presence of minor amounts of a number of branched-chain acids and acids with an odd number of carbon atoms (14-20, 36-38).

The identification and structure of the unsaturated fatty acids of butterfat have not been completely elucidated and are still the subject of extensive investigation. As early as 1912, Smedley (40) suggested that lower homologs of oleic acid were present. Other workers (5, 11, 12, 27) substantiated Smedley's postulation for the presence of decenoic acid and found evidence of  $C_{12}$ ,  $C_{14}$ , and  $C_{16}$  monoethenoid acids. The double bond was claimed to be in the 9, 10 position (26, 29). Bertram's (4) discovery, in 1928, of vaccenic (*trans*-11-octadecenoic) acid led to further studies by others (2, 3, 7, 9, 13, 33) who also concluded that octadecenoic acids contained position as well as geometric isomers. Until recently, the lower unsaturated acids were thought to be exclusively *cis* forms. However, in 1954, Smith and coworkers (47) presented evidence of *trans* components in  $C_{12}$ ,  $C_{14}$ ,  $C_{16}$ , and  $C_{18}$  monoethenoid fatty acid fractions and in 1956, Backderf (2) found a *trans*-16-octadecenoic acid, a previously unreported component of butterfat.

Some early investigators concluded that the diethenoid acids of butterfat were mainly geometrical isomers of linoleic acid (5, 6, 8, 24, 25), while others found that these acids contained a high percentage of normal linoleic (9-*cis*, 12-*cis* octadecadienoic) acid (44). In 1954, Smith and coworkers (47) pub-

lished the infrared spectra of concentrates of polyunsaturated acids from butterfat, which showed the conjugated dienoic acid to be largely *cis-trans* with the possibility that some *trans-trans* isomers were also present.

Substantially all the nonconjugated trienoic acid of butterfat has been identified as normal linolenic (9-*cis*, 12-*cis*, 15-*cis* octadecatrienoic) acid by the preparation of the bromo derivatives (10, 35).

Ultraviolet spectrophotometric evidence of a tetraenoic and a pentaenoic acid has been published (30-32, 39, 42). However, these acids have not been isolated and characterized.

The present study was initiated primarily to identify the polyunsaturated components of butterfat. However, in the course of the work, the monoethenoid components were also investigated. Owing to the inadequacies of the analytical methods for such complex mixtures containing geometric isomers, no attempt was made to relate quantitatively the amount of each unsaturated component in the various fractions to the amount in the butterfat.

### Experimental

The butterfat employed in this investigation was prepared from butter churned from fresh pasteurized cream. The milk was obtained from the dairy herd located at the U. S. Department of Agriculture Research Center, Beltsville, Md. The herd had been fed winter rations for several months.

As many of the unsaturated fatty acids are present in minor proportions, it was necessary to concentrate them to facilitate their isolation. Four kilograms of butterfat were converted directly to the methyl esters by methanolysis; sodium methylate was used as catalyst. The methyl esters were distilled under reduced pressure to remove esters up

to and including some of the esters of the  $C_{16}$  acids. The remainder were subjected to a series of crystallizations from acetone at  $-30^\circ\text{C}$ .,  $-45^\circ\text{C}$ ., and  $-65^\circ\text{C}$ . to remove most of the palmitate, stearate, and some of the octadecenoate. The  $-65^\circ\text{C}$ . filtrate material, 605 grams, was subjected, in 80- to 100-gram batches, to further fractionation by chromatographic adsorption on silicic acid columns. The column and technique for operation under nitrogen were essentially the same as described by Herb and Riemenschneider (22, 23, 34). The adsorbent mixture, 80% silicic acid-20% filter aid, was heated to  $105^\circ\text{C}$ . for 16 hours prior to packing in the column as a slurry in redistilled petroleum ether. The column was 6.5 cm. in diameter and 105 cm. in height and was packed with 1500 to 1600 grams of adsorbent mixture to a depth of 90 to 94 cm. Successive elutions were made with redistilled petroleum ether (boiling point  $40-50^\circ\text{C}$ .) containing increasing percentages of ethyl ether depending on the progress of the fractionation, which was determined by analysis of the fractions.

Fractions from this large column having similar composition, whether from the same or separate runs, were combined for further fractionation either by adsorption on a smaller silicic acid column, low pressure distillation, or urea-adduct stepwise crystallization. The smaller chromatographic adsorption columns were of the same general type as the larger column and were packed so that there was a ratio of 30 or more grams of silicic acid-filter aid mixture per gram of sample.

The urea adduct stepwise crystallization procedure was the same as that described by Allen (7). Fifteen grams of the esters were dissolved in 150 ml. of methanol, 15 grams of urea was added, and the mixture heated until the urea dissolved. The solution was held

**Table I. Spectrophotometric Analysis of Butterfat and Concentrate from Butterfat**

Acids	Butterfat (I.V. 30.2) Acid in Sample, %	Concentrate (I.V. 101.0)
Conjugated		
Dienoic	0.43	2.23
Trienoic	0.02	0.07
Nonconjugated		
Dienoic	1.50	8.70
Trienoic	0.59	3.70
Tetraenoic	0.23	1.40
Pentaenoic	0.20	1.20
Monoenoic	29.1	68.80
Saturated (by difference)	65.9	8.90

at room temperature until the complex crystallized; the complex was removed by filtration. The filtrate was treated with 12 grams of urea to remove another fraction of the esters. The urea treatments were repeated until all the esters were recovered.

**Methods of Analyses.** The iodine values were determined by the Wijs method (30 minutes). Polyunsaturated acids were determined by the micro method of Herb and Riemenschneider (27), using a Beckman Model DU spectrophotometer. Infrared data were obtained with a Perkin-Elmer Model 21 spectrophotometer using essentially the procedure of Swern *et al.* (43).

#### Interpretation of Infrared Data.

The infrared region from 900 to 1100  $\text{cm}^{-1}$  (11.11 to 9.09 microns) is shown in the figures. The pertinent bands for interpretation of geometrical configuration are at 948, 968, 982, and 988  $\text{cm}^{-1}$  (10.55, 10.33, 10.18, and 10.12 microns). The 968- $\text{cm}^{-1}$  band is characteristic of a trans bond in a mono unsaturated ester or of a trans bond in a nonconjugated polyunsaturated ester. In this paper, the former will be referred to as an isolated trans bond and the latter as a nonconjugated trans bond. The band at 988  $\text{cm}^{-1}$  and the doublet at 948 and 982  $\text{cm}^{-1}$  were established by Jackson and coworkers (28) as characteristic of trans-trans conjugated diene and cis-trans conjugated diene, respectively. Unresolved absorption in the 982 to 988- $\text{cm}^{-1}$  region in conjunction with a definite band at 948  $\text{cm}^{-1}$  was considered evidence of both cis-trans and trans-trans conjugated diene.

The infrared curves are in per cent transmittance. The spectra of different fractions are presented in order to show the relative effect of various techniques in fractionating mixtures containing geometric isomers of the unsaturated components. The intensity of the trans bands shown in the curves for the fractions represent only visual comparisons of the trans components in these frac-

tions and are not intended to show a quantitative interrelationship.

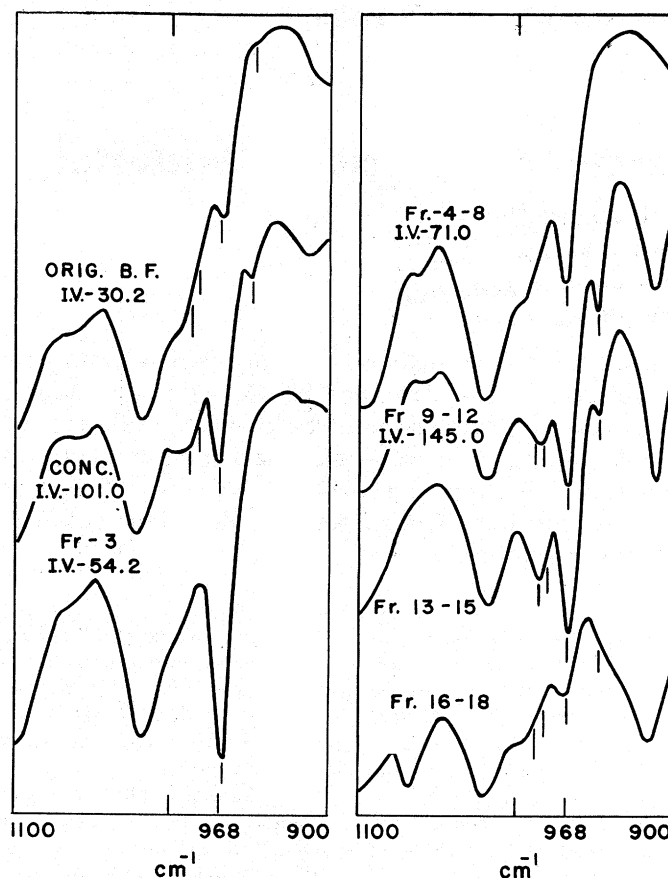
In every investigation where a number of fractionating techniques and manipulations are applied to unsaturated fatty acids and esters there is always the possibility of artifacts being formed. Precautions were exercised at every stage of the separation to minimize this possibility by preventing exposure to air and excessive heating. In no instance in the course of employing fractionating procedures for obtaining enrichment of the components was there observed an unexpected increase in trans components as might be the case if they were being produced as artifacts. Hence, the trans material did not appear to result from manipulative or chemical procedures. Likewise, other workers (9, 47) found no evidence that similar techniques produced trans isomers as artifacts.

#### Results and Discussion

Further fractionation of the low pressure distillates or the precipitates from the fractional crystallization has not been attempted here. However, iodine values, saponification equivalents, and ultraviolet and infrared spectrophotometric analyses were obtained on these

products. These analyses indicated that the unsaturated constituents of the distillates were principally esters of  $\text{C}_{10}$ ,  $\text{C}_{12}$ ,  $\text{C}_{14}$ , and  $\text{C}_{16}$  monoene acids, the esters of  $\text{C}_{12}$  and  $\text{C}_{14}$  acids being predominantly of the cis configuration. The major components of the precipitates from the fractional crystallization were methyl palmitate, methyl stearate, and methyl oleate.

Principal attention was given to the identification of the unsaturated components of the filtrate obtained from the  $-65^\circ\text{C}$ . crystallization. The ultraviolet spectrophotometric analysis of this filtrate material ( $-65^\circ\text{C}$ . concentrate) and of the original butterfat are given in Table I. The infrared spectra are shown in Figure 1. The original butterfat contained small amounts of conjugated diene and traces of conjugated triene esters. Nonconjugated esters with 2, 3, 4, and 5 double bonds were present. The total polyunsaturated acids present in butterfat is about 3%. A small amount of isolated or nonconjugated trans isomers are evident from the infrared spectrum. This is estimated to be not more than 5%. The  $-65^\circ\text{C}$ . concentrate material represented about a sixfold concentration of polyunsaturated esters.



**Figure 1. Infrared curves of butterfat methyl esters, concentrate of unsaturated esters, and fractions from a chromatographic separation of the concentrate. Ordinate is transmittance with zero displaced**

**Table II. Analyses of Fractions from Chromatographed Concentrate**

Fraction	Wt., G.	I.V.	Conjugated Diene	Nonconjugated			
				Diene	Triene	Tetraene	Pentaene
				Acid in Sample, %			
1-2	0	...	...	...	...	...	...
3	6.3	54.2	0.003	TR.	...	...	...
4-5	17.9	70.6	0.004	TR.	...	...	...
6-7-8	24.0	71.0	0.009	0.85	...	...	...
9	15.3	79.6	1.05	2.41	...	...	...
10	10.6	114.4	3.96	23.9	...	...	...
11	9.1	149.8	7.16	56.2	0.35	...	...
12	1.7	161.1	4.59	55.7	2.72	...	...
13	0.4	...	11.2	34.3	8.20	...	...
14	0.2	...	15.5	39.8	22.7	...	...
15	1.1	...	...	14.3	48.0	9.0	...
16	1.9	...	...	...	63.5	17.9	...
17	1.0	...	...	...	68.2	24.7	...
18	1.5	296.6	2.66	...	7.7	11.4	35.0
Residue	9.5	Viscous material containing 13% unsap.					

The infrared spectrum of the concentrate showed the presence of conjugated cis-trans and trans-trans isomers and an effective enrichment of the isolated or nonconjugated trans compounds as compared with the original butterfat.

The analyses and infrared spectra of the fractions from a typical chromatographic separation of the concentrate are illustrated in Table II and Figure 1. Fraction 3 contained saturated and monoene ester while fractions 4 to 8 consisted mainly of monoene esters with a lesser amount of saturated esters. The infrared curves show that fraction 3 contained a greater concentration of isolated trans bonds than fractions 4 to 8. This suggests that some fractionation of the trans from the cis monoene esters had resulted.

Ultraviolet spectrophotometric analysis of fractions 9 to 12, both before and after isomerization, showed the presence of conjugated and nonconjugated diene esters and some nonconjugated triene esters. The infrared spectrum confirmed the presence of cis-trans and suggested the possible presence of trans-trans conjugated diene esters and nonconjugated diene containing some trans double bonds. The probable presence of trans-trans conjugated diene is more clearly shown by the infrared curves for fractions 13 to 15, which also contained a small amount of nonconjugated tetraene esters as indicated by the ultraviolet analysis.

Examination of the infrared spectra of fractions 9 to 12 and 13 to 15 shows that the unresolved absorption at 982 to 988  $\text{cm}^{-1}$  favors the 982  $\text{cm}^{-1}$  band in fraction 9 to 12 and the 988  $\text{cm}^{-1}$  band in fraction 13 to 15. This, when considered with the intensity of the 948  $\text{cm}^{-1}$  band in their respective spectra, indicates that some fractionation of the conjugated cis-trans from the conjugated trans-trans isomers has been achieved. Further evidence for the separation of these isomers is shown by the relative amounts of conjugated dienes removed in fractions 9 to 14

(Table II). Fractions 16 to 18 consisted essentially of triene, tetraene, and pentaene esters having the all-cis configuration, as the small band at 968  $\text{cm}^{-1}$  represents less than 3% trans material calculated as methyl elaidate.

**Separation of Cis and Trans  $\text{C}_{18}$  Monoene Esters.** A composite of fractions similar in composition to fraction 3 (Table II) was rechromatographed. The infrared spectra are shown in Figure 2. Fractions A and B, which are essentially all saturated as indicated by the iodine values, contained only a slight amount of trans material. Fraction C had a high percentage of isolated trans isomers, while Fraction D, which has an iodine value close to that of methyl oleate 85.1 (theory 85.6), contained no trans isomers. This shows the feasibility of this procedure for separating trans from cis monoene esters.

**Separation of Trans-Trans and Cis-Trans Diene Esters.** A sample of esters, which had an analysis similar to that of the composite of fractions 9 to 12 (Table II), was subjected to urea-adduct stepwise crystallization. The infrared curve of the composite is designated O in Figure 3. The conjugated trans-trans isomers were found mainly in precipitate fraction A, while the conjugated cis-trans isomers were found predominantly in precipitate fraction C. Precipitate fraction B and the final filtrate D contained very small amounts of both. Most of the isolated or nonconjugated trans material concentrated in fractions A and B. The final filtrate D, by ultraviolet spectrophotometric analysis, was rich in dienes; hence, even though there is evidence of small amounts of trans isomers, the presence of cis-cis nonconjugated linoleate is almost certain.

**Separation of Triene and Tetraene Esters.** Attempts to fractionate material comparable to fractions 16 and 17 (Table II) were only partially successful. Rechromatographing produced fractions containing 88 to 89% triene and 7 to

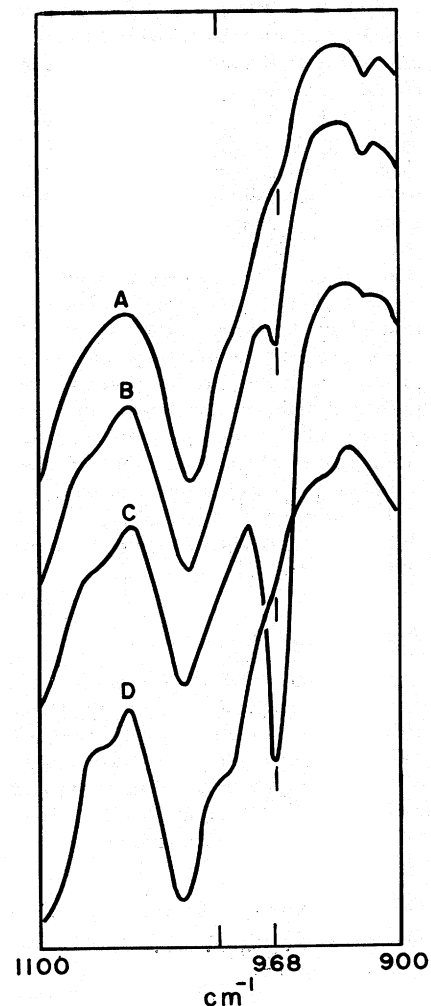


Figure 2. Infrared curves demonstrating the fractionation of butterfat cis monoethenoid from trans monoethenoid esters. Ordinate is transmittance with zero displaced

- A. I.V. 8.0, mostly saturated esters
- B. I.V. 8.7, mostly saturated esters
- C. I.V. 62.8, saturated + cis and trans methyl oleate
- D. I.V. 85.1, essentially pure cis methyl oleate

8% tetraene esters with no trans bonds, and fractions containing about 45% tetraene and 49% triene esters which had no trans bonds. The x-ray diffraction pattern of a completely hydrogenated portion of a fraction, which contained a high percentage of triene, showed only the presence of esters of  $\text{C}_{18}$  acids. Similar treatment of a fraction which contained a high percentage of tetraene indicated that the tetraene was an ester of a  $\text{C}_{22}$  acid although the presence of a  $\text{C}_{20}$  tetraene was not excluded.

**Pentaene Esters.** A composite of fractions comparable to fraction 18 (Table II) was rechromatographed. A fraction was obtained that appeared to be an ester of a  $\text{C}_{22}$  pentaenoic acid as judged by the iodine value, 368.3 (theory 368.5), and from a hydrogen number of 67.71 (iodine equivalent 374.8). However, the absorptivity determined by

ultraviolet spectrophotometric analysis was high for this ester,  $a_{346\text{ m}\mu} = 56.4$ , an indication that the fraction probably contained the esters of both  $C_{22}$  and  $C_{20}$  pentaenoic acids [published values (27)  $a_{346\text{ m}\mu} = 48.3$  and  $83.6$ , respectively]. From the spectrophotometric data, the fraction was estimated to contain 77% esters of  $C_{22}$  and 23% esters of  $C_{20}$  pentaenoic acids. The calculated iodine number of such a mixture would be 376.0, which is in good agreement with that found by hydrogenation.

An x-ray diffraction study of a completely hydrogenated portion of this particular fraction and known mixtures indicated the presence of more than two components, which made the interpretation of chain length inconclusive.

The infrared spectrum, Figure 4, indicated that the pentaenes had the all-cis configuration.

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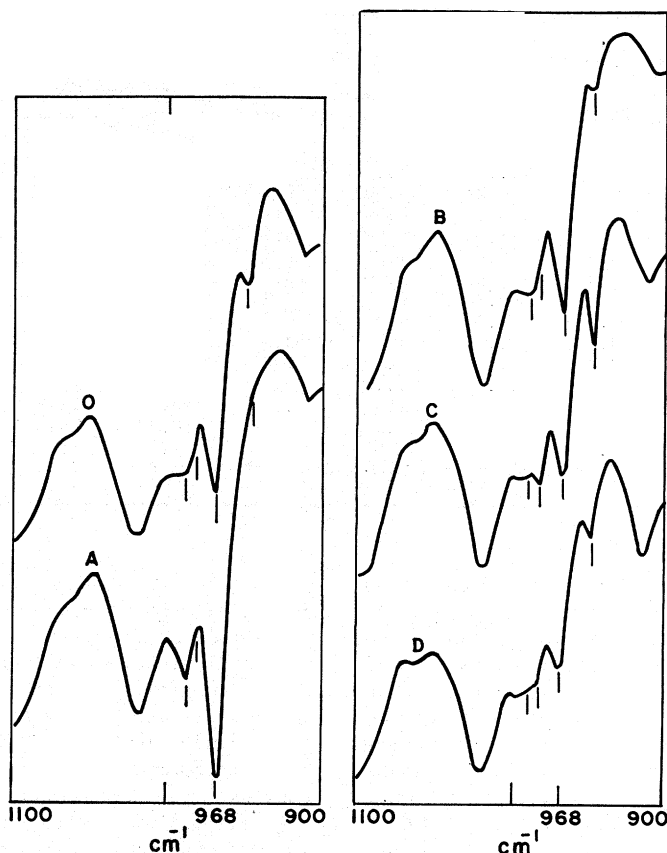


Figure 3. Infrared curves demonstrating the separation of cis-trans from trans-trans conjugated methyl linoleate by step-wise urea adduct crystallization. Ordinate is transmittance with zero displaced

- O. Fraction similar to Fr. 9-12 (Table II)
- A. 1st ppt. fraction
- B. 2nd ppt. fraction
- C. 3rd ppt. fraction
- D. Final filtrate

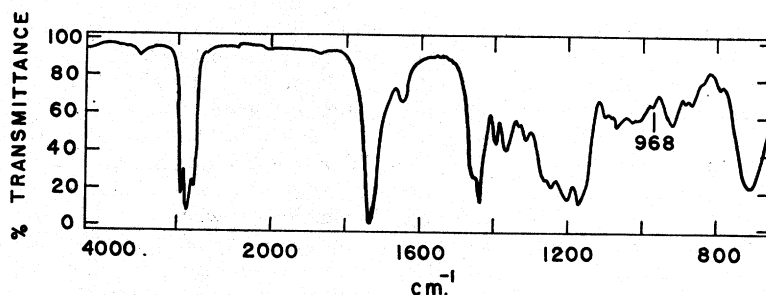


Figure 4. Infrared curve of methyl pentaenoate from butterfat. Determined as a liquid

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